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(54) Title: PROTECTION OF ENDOGENOUS THERAPEUTIC PEPTIDES FROM PEPTIDASE ACTIVITY THROUGH CONJUGATION TO BLOOD COMPONENTS

(57) Abstract

A method for protecting a peptide from peptidase activity *in vivo*, the peptide being composed of between 2 and 50 amino acids and having a C-terminus and an N-terminus and a C-terminus amino acid and an N-terminus amino acid is described. In the first step of the method, the peptide is modified by attaching a reactive group to the C-terminus amino acid, to the N-terminus amino acid, or to an amino acid located between the N-terminus and the C-terminus, such that the modified peptide is capable of forming a covalent bond *in vivo* with a reactive functionality on a blood component. In the next step, a covalent bond is formed between the reactive group and a reactive functionality on a blood component to form a peptide-blood component conjugate, thereby protecting said peptide from peptidase activity. The final step of the method involves the analyzing of the stability of the peptide-blood component conjugate to assess the protection of the peptide from peptidase activity.

Relevant pages only.

total pages 433

dimethylformamide (DMF) and 3 times with isopropanol. The peptide is cleaved from the resin using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold Et2O (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in H2O (A) and 0.045% TFA in CH3CN (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in >95% purity, as determined by RP-HPLC.

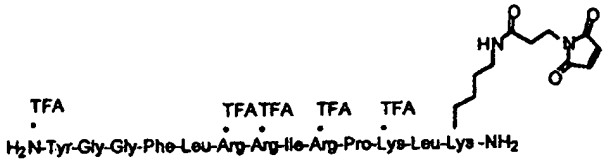
**Example 8 – Modification of Dyn A 1-13 at the ε-Amino Group of the Added C-terminus Lysine Residue - Synthesis of Dyn A 1-13(Nε-MPA)-$NH_2$**

**Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-(Nε-MPA)-$NH_2$**

Solid phase peptide synthesis of a modified Dyn A 1-13 on a 100 μmole scale was performed using manual solid-phase synthesis, a Symphony Peptide Synthesizer and Fmoc protected Rink Amide MBHA. The following protected amino acids were sequentially added to resin: Fmoc-Lys(Aloc)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Tyr(tBu)-OH. They were dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (V/V) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (Step 1).The selective deprotection of the Lys (Aloc) group is performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin is then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5

mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis is then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Between every coupling, the resin is washed 3 times with *N,N*-dimethylformamide (DMF) and 3 times with isopropanol. The peptide is cleaved from the resin using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold $Et_2O$ (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in $H_2O$ (A) and 0.045% TFA in $CH_3CN$ (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in >95% purity, as determined by RP-HPLC.

The structure of this product is



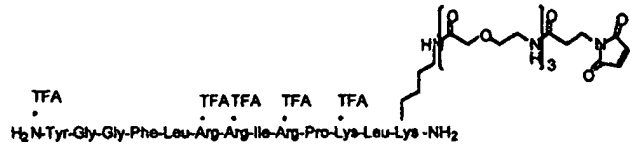

**Example 9 - Modification of Dyn A 2-13 at the ε-Amino Group of the Added C-terminus Lysine Residue - Synthesis of Dyn A 2-13(Nε-MPA)-$NH_2$**
**Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-(Nε-MPA)-$NH_2$**

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Mtt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, and Boc-Gly-OH. Manual synthesis was employed for the remaining steps: selective removal of the Mtt group and coupling of MPA using HBTU/HOBt/DIEA activation in

DMF. The target dynorphin analog was removed from the resin; the product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization in a 35% yield. Anal. HPLC indicated product to be >95% pure with $R_t$ = 30.42 min. ESI-MS *m/z* for $C_{73}H_{123}N_{25}O_{15}$ ($MH^+$), calcd 1590.0, found $MH^{3+}$ 531.3.

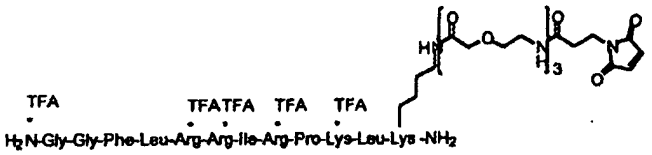
**Example 10 - Modification of Dyn A 1-13 at the ε-Amino Group of the Added C-terminus Lysine Residue - Synthesis of Dyn A 1-13($AEA_3$-MPA)-$NH_2$**
**Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-($AEA_3$-MPA)-$NH_2$**

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Mtt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, and Boc-Tyr(Boc)-OH. Manual synthesis was employed for the remaining steps: selective removal of the Mtt group, the coupling of three-Fmoc-AEA-OH groups (AEA = aminoethoxyacetic acid) with Fmoc removal in-between each coupling, and MPA acid using HBTU/HOBt/DIEA activation in DMF. The target dynorphin analog was removed from the resin; the product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization in a 29% yield. Anal. HPLC indicated product to be >95% pure with $R_t$ = 33.06 min. ESI-MS *m/z* for $C_{94}H_{154}N_{29}O_{23}$ ($MH^+$), calcd 2057.2, found $MH^{4+}$ 515.4, $MH^{3+}$ 686.9, $MH^{2+}$ 1029.7.



**Example 11 – Modification of Dyn A 2-13 at the ε-Amino Group of the Added C-terminus Lysine Residue - Synthesis of Dyn A 2-13(AEA$_3$-MPA)-NH$_2$**
**Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-(AEA$_3$-MPA)-NH$_2$**

Using automated peptide synthesis, the following protected amino acids were sequentially added to Rink Amide MBHA resin: Fmoc-Lys(Mtt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, and Fmoc-Gly-OH. Manual synthesis was employed for the remaining steps: selective removal of the Mtt group, the coupling of three-Fmoc-AEA-OH groups, with Fmoc removal in-between each coupling, and MPA using HBTU/HOBt/DIEA activation in DMF. The target dynorphin analog was removed from the resin; the product was isolated by precipitation and purified by preparative HPLC to afford the desired product as a white solid upon lyophilization in a 29% yield. Anal. HPLC indicated product to be >95% pure with $R_t$ = 31.88 min. ESI-MS *m/z* for $C_{85}H_{145}N_{25}O_{21}$ ($MH^+$), calcd 1894.3, found $MH^{4+}$ 474.6, $MH^{3+}$ 632.4, $MH^{2+}$ 948.10.




**Example 12 – Modification of Neuropeptide Y at the ε-Amino Group of the Added C-terminus Lysine Residue**
**Preparation of Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Lys-(N-εMPA)-NH$_2$**

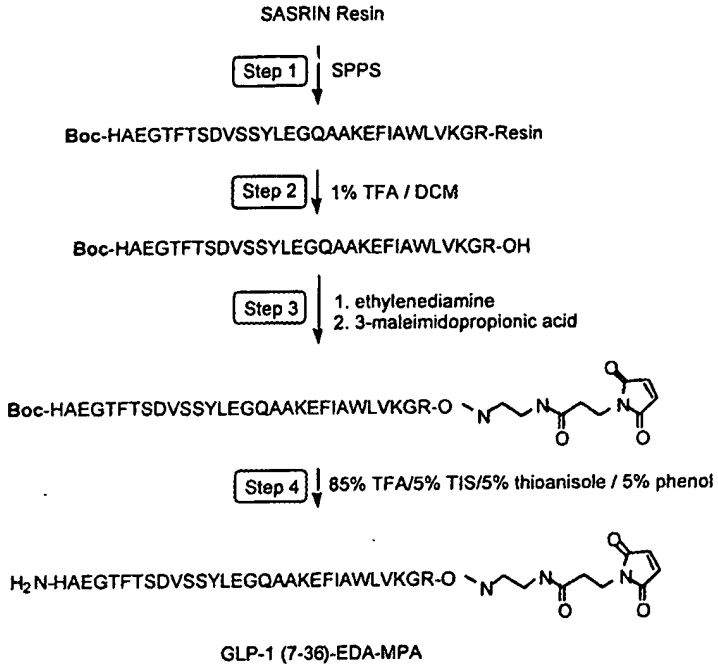
Solid phase peptide synthesis of a modified neuropeptide Y analog on a 100 µmole scale is performed using manual solid-phase synthesis, a Symphony Peptide Synthesizer and Fmoc protected Rink

Amide MBHA. The following protected amino acids were sequentially added to resin: Fmoc-Lys(Aloc)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Ser(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Met-OH, Fmoc-Asp(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Asp(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Tyr(tBu)-OH,They are dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (*V/V*) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (Step 1).The selective deprotection of the Lys (Aloc) group is performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin is then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis is then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Between every coupling, the resin is washed 3 times with *N,N*-dimethylformamide (DMF) and 3 times with isopropanol. The peptide is cleaved from the resin using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold $Et_2O$ (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in $H_2O$ (A) and 0.045% TFA in $CH_3CN$ (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in >95% purity, as determined by RP-HPLC.

**Example 13 - Modification of GLP-1 (7-36) at the C-Terminus Arginine**
**Preparation of GLP-1 (7-36)-EDA-MPA**

Solid phase peptide synthesis of a modified GLP-1 analog on a 100 µmole scale is performed manually and on a Symphony Peptide Synthesizer SASRIN (super acid sensitive resin). The following protected amino acids are sequentially added to the resin: Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ala-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmco-Glu(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Asp(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ala-OH, Boc-His(Trt)-OH. They are dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (V/V) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (Step 1). The fully protected peptide is cleaved from the resin by treatment with 1% TFA / DCM (Step 2). Ethylenediamine and 3-maleimidopropionic acid are then sequentially added to the free *C*-terminus (Step 3). The protecting groups are then cleaved and the product isolated using 86% TFA/5% TIS/5% $H_2O$/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold $Et_2O$ (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax $C_{18}$, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax $C_{18}$, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in

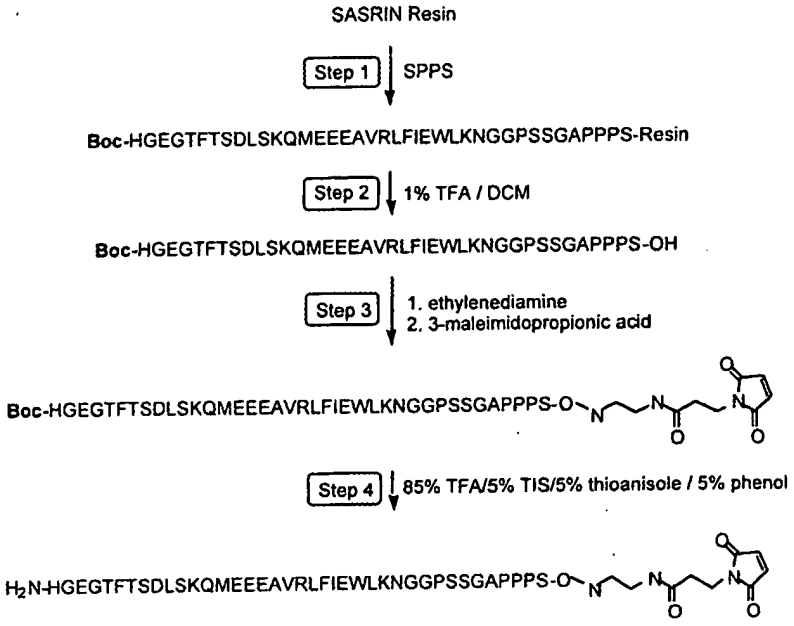
>95% purity, as determined by RP-HPLC. These steps are illustrated in the schematic diagram below.




**Example 14 - Modification of Exendin-4 at the C-terminus Serine**
**Preparation of Exendin-4 (1-39)-EDA-MPA**

Solid phase peptide synthesis of a modified Exendin-4 analog on a 100 µmole scale is performed manually and on a Symphony Peptide Synthesizer SASRIN (super acid sensitive resin). The following protected amino acids are sequentially added to the resin: Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Asn(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-

OH, Fmoc-Phe-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Boc-His(Trt)-OH. They are dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (V/V) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (Step 1). The fully protected peptide is cleaved from the resin by treatment with 1% TFA / DCM (Step 2). Ethylenediamine and 3-maleimidopropionic acid are then sequentially added to the free *C*-terminus (Step 3). The protecting groups are then cleaved and the product isolated using 86% TFA/5% TIS/5% $H_2O$/2% thioanisole and 2% phenol, followed by precipitation by dry-ice cold $Et_2O$ (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system using a Dynamax $C_{18}$, 60Å, 8 µm, 21 mm x 25 cm column equipped with a Dynamax $C_{18}$, 60Å, 8 µm guard module, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in >95% purity, as determined by RP-HPLC.




Exendin-4 (1-39)-EDA-MPA

**Example 15 – Modification of Secretin Peptide at the ε-Amino Group of the Added C-terminus Lysine Residue Preparation of His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Glu-Arg-Leu-Leu-Gln-Gly-Leu-Val-Lys-(Nε-MPA)-$NH_2$**

Solid phase peptide synthesis of a modified secretin peptide analog on a 100 μmole scale is performed using manual solid-phase synthesis, a Symphony Peptide Synthesizer and Fmoc protected Rink Amide MBHA. The following protected amino acids are sequentially added to resin: Fmoc-Lys(Aloc)-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Glu(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Glu(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-His(Boc)-OH.They are dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (V/V) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (Step 1).The selective deprotection of the Lys (Aloc) group is performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin is then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis is then re-automated for the addition of the 3-maleimidopropionic acid (Step 3). Between every coupling, the resin is washed 3 times with *N,N*-dimethylformamide (DMF) and 3 times with isopropanol. The peptide is

cleaved from the resin using 85% TFA/5% TIS/5% thioanisole and 5% phenol, followed by precipitation by dry-ice cold $Et_2O$ (Step 4). The product is purified by preparative reverse phase HPLC using a Varian (Rainin) preparative binary HPLC system: gradient elution of 30-55% B (0.045% TFA in $H_2O$ (A) and 0.045% TFA in $CH_3CN$ (B)) over 180 min at 9.5 mL/min using a Phenomenex Luna 10 μ phenyl-hexyl, 21 mm x 25 cm column and UV detector (Varian Dynamax UVD II) at λ 214 and 254 nm to afford the desired DAC in >95% purity, as determined by RP-HPLC.

**Example 16 – Modification of Kringle-5 at the ε-Amino Group of the Added C-terminus Lysine Residue**
**Preparation of NAc-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-Lys-(Nε-MPA)-$NH_2$.2TFA**

Solid phase peptide synthesis of a modified Kringle-5 peptide on a 100 μmole scale was performed using manual solid-phase synthesis, a Symphony Peptide Synthesizer and Fmoc protected Rink Amide MBHA. The following protected amino acids are sequentially added to resin: Fmoc-Lys(Aloc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. They were dissolved in *N,N*-dimethylformamide (DMF) and, according to the sequence, activated using O-benzotriazol-1-yl-*N*, *N*, *N'*, *N'*-tetramethyl-uronium hexafluorophosphate (HBTU) and Diisopropylethylamine (DIEA). Removal of the Fmoc protecting group is achieved using a solution of 20% (*V/V*) piperidine in *N,N*-dimethylformamide (DMF) for 20 minutes (step 1). At the end of the synthesis Acetic Anhydride was added to acetylate the N-terminal.The selective deprotection of the Lys (Aloc) group is performed manually and accomplished by treating the resin with a solution of 3 eq of $Pd(PPh_3)_4$ dissolved in 5 mL of $CHCl_3$:NMM:HOAc (18:1:0.5) for 2 h (Step 2). The resin is then washed with $CHCl_3$ (6 x 5 mL), 20% HOAc in DCM (6 x 5 mL), DCM (6 x 5 mL), and DMF (6 x 5 mL). The synthesis is then re-automated for the addition of the 3-maleimidopropionic acid (Step 3).